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Bezeichnung: Color coded nanoparticles

IPC: B 01 J, C 08 J, G 01 N

Die angehefteten Stücke sind eine richtige und genaue Wiedergabe der ursprünglichen Unterlagen dieser Patentanmeldung.

München, den 6. August 2003
Deutsches Patent- und Markenamt
Der Präsident
Im Auftrag

Klostermeyer

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Color-coded nanoparticles

Claims

1. Composition for the identification of materials, the composition comprising at least two species of capsules with diameters of less than 100 μm, wherein the capsules have a core and a shell, and wherein the shell comprises at least three layers, and wherein at least one of said layers is labeled with a dye.
2. The composition of claim 1, comprising at least 3 species of capsules.
3. The composition of claim 1 or 2, wherein the capsules have mean diameters of less than about 10 μm, more preferably less than about 1 μm.
4. The composition of any of the preceding claims, wherein said shells are composed of layers of polyelectrolytes.
5. The composition of any of the preceding claims, wherein at least one of the capsule species is defined by capsules whose shells comprise at least two layers which are labeled with different dyes, said layers being separated from each other by at least one non-labeled layer.

Color-coded nanoparticles

Description

The present invention deals with a combinatorial library on the base of hollow or filled polyelectrolyte capsules prepared by the layer by layer method. The LbL method enables the control of the number, the concentration and the distance between the dye molecules on the nanometer scale which results in more coding information in the wall as it is known from beads which are color coded in the volume or on the surface. The second part of the invention deals with the opportunity to fill the capsules with different macromolecules but to keep the capsules still permeable for small molecules. Such color-coded capsules can be used as combinatorial fishing containers, which can uptake remarkable amounts of specific compounds from a reaction mixture. Afterwards the different capsules with the different compounds in the interior can be sorted out by means of their specific fluorescence signals. These combinatorial libraries can be applied in many fields of medicine, biology and chemistry.

Assay miniaturization and microtiterplate logistic are limited for increasing assay capacity. An alternative method is offered by bead based libraries. New developments in Flow Cytometry (e.g. COPAS™ bead flow sorting) allows a throughput up to 100 000 compounds per hour. Hence, bead based libraries may become the leading technology in screening or fishing operations.[1-5, 7]

We have prepared hollow capsules from polyelectrolytes[6] which contains different color combinations in the wall. The color-coded capsules can be sorted like beads but they are hollow and could contain many reaction sites both on the wall surface but also in the interior.

These capsules have several advantages compared to the bead technology:

1. Their mass is very low. Hence in solvents with different specific density they precipitate much slower than beads.
2. Due to the thin wall and similar or same material inside like outside the scattering of light is very small. In case of beads differences in the refractive index between bead and solvent (usual water) strong light scattering occur perturbing the sorting process in the flow cytometer.

3. Reactions on beads are possible only on the surface. Hence the number of reaction sites is strongly limited. In case of our capsules the outer wall surface, the inner wall surface and the whole volume of the capsule can be used for reactions. A capsule (or bead) of 5 μm diameter have an outer surface of 78 μm^2 and a volume of 65 μm^3. Assuming a 0.1 M concentration of reaction sites, one bead has approx. only 9×10^4 reaction sites whereas one capsule has 5000 times more namely 4×10^8 reaction sites.
4. The dye labels can be controlled deposited with enough space between them in order to avoid interactions like formation of H- or J-aggregates, self-quenching, or Förster Resonance Energy Transfer which perturb the fluorescence signals in case of labelling bulk phases with several different dyes. This allows more combinatorial opportunities.
5. Förster Resonance Energy Transfer signals can be controlled induced for forgery-proof coding of trade-marks.
6. The interior of the capsules can be filled with highly active bio-agents like enzymes DNA etc. or with specific functionalised polyelectrolytes allowing selective fishing of reaction partners from solution by rbio-reactions, physic- or chemisorption. Subsequently the coded capsules can be sorted out.
7. The encoding information can be obtained by the number of dyes, their ratio to each other and also by distance dependent interactions between them such as Förster Resonance Energy Transfer. In known fluorescent beads[4] such interactions are undesired because the distance between the dye molecules can not be controlled.
8. The preparation of hollow coded capsules and the use of the interior for the immobilization of macromolecules (polyelectrolytes, proteins, enzymes). The functionalised macromolecules can fish complementary compounds from reaction solution by physisorption, chemisorption or biological binding.

Description of the experiments:

Dye labelling of polyelectrolytes: PAH was labelled with the dye derivatives fluoresceine isothiocyanate, tetramethylrhodamine isothiocyanate and a derivative of Cy5. The formulas are shown in Figure 1. The label reaction was performed according general procedures in protein labelling. Instead of a hydrogencarbonate buffer NaOH has been used for the activation of about 30% of the PAH groups. The reaction solutions were dialized against water. After HCl had been added to the labelled PAH solution until a pH of 4-5 had been reached the solution were lyophilised. The label contents were determined by UV/Vis

spectroscopy for PAH-Fl to 53:1, for PAH-Rho 580:1 and for PAH-Cy5 500:1 (ratio PAH unit: number of dye molecules). The yield of labelling amounts to 80% for fluorescein, to 20% for rhodamine and to 40% for Cy5. Each PAH was labelled only by one dye because simultaneous labelling at one PAH chain has the drawback of self-quenching or Förster resonance energy transfer.

The absorption and fluorescence spectra of the dyes are shown in Fig. 2 a and b, respectively. The absorption maxima of the three labelled PAH were measured to be 495, 557, 648 nm, respectively. The fluorescence maxima are 520, 582, 665 nm, using the absorption wavelengths for the excitation.

Capsule preparation:

3 µm silica templates were coated by 10 alternating layers of poly(allylamine hydrochloride) (PAH, MW 60 000 g/mol and of poly(styrenesulfonate) (PSS, MW 70 000 g/mol).[9] In order to obtain distinguishable walls, differently labelled PAH polymers have been used for the deposition. For the coloration of the capsules only one layer of the corresponding PAH has been assembled. Only in case of the Cy5 two layers were prepared because of the lower fluorescence quantum yield and the low label content of the PAH-Cy5. It was tried to keep some distance between different dye layers in order to avoid Förster Resonance Energy Transfer. Following capsules were prepared:

Table 1: Capsules, coloured with different kinds of PAH-dye layers

Layer/capsule	1.	2.	3.	4.	5.	6.	7.	8.
1. PAH	-	-	-	-	-	-	-	-
2. PSS	-	-	-	-	-	-	-	-
3. PAH	-	-	-	Cy5	Cy5	Cy5	Cy5	-
4. PSS	-	-	-	-	-	-	-	-
5. PAH	Rho	Rho	Fluo	Cy5	Cy5	Cy5	Cy5	-
6. PSS	-	-	-	-	-	-	-	-
7. PAH	-	-	-	-	Fluo	Rho	Fluo	-
8. PSS	-	-	-	-	-	-	-	-
9.PAH	-	Fluo	-	-	-	-	Rho	-
10. PSS	-	-	-	-	-	-	-	-

After dissolution of the silica core by hydrofluoric acid and washing by water hollow capsules were obtained.

The capsules were investigated by confocal laser scanning microscopy using three different channels simultaneously (Fig. 3a-c). The excitation wavelengths of the lasers were 488 nm for the fluorescein dye, 543 nm for the rhodamine dye, and 633 nm for the Cy5 dye. The detectors were settled according the maximal emission of the dyes and a minimal overlap of their fluorescence emission. The laser intensities and detector sensitivities were adjusted according to comparable fluorescence signals for each channel. The overlay of the 3 channels yielded 7 differently coloured capsules (Fig. 3d).

A quantitative and save method for distinguishing the capsules offered the analysis of the profiles through the capsules. The profiles exhibit the distribution of fluorescence intensities on different channels for the same capsule. Fig. 4a shows for example the profile of capsules 2, 7, 1, and 5.

The fluorescence intensity per dye layer is not the same for the differently coloured capsules which might be caused by resonance energy transfer effects as well as by different amounts of adsorbed material. The resonance energy transfer can be reduced remarkably by using more layers in between the dye layers. Above a distance of 6 nm (approx. 4 layers) almost no perturbation occurs between the dye molecules.

Controlled Förster Resonance energy transfer[10]

In order to use fixed distances between the dye molecules for protecting trademarks for falsification, capsules with different distance but same content of dyes have been prepared. Figure 5 shows the prepared layer combinations.

The information in the capsules can be encoded for two dyes by using two different wavelengths for the excitation and measure the fluorescence at two different wavelengths. In case of the Rhodamine/ Fluoresceine system that is:

1. Excitation light 540 nm, emission at 576 nm: yielded absolute concentration of rhodamine
2. Excitation light 495 nm, emission at 520 nm: yielded concentration of fluoresceine minus the concentration of the molecules undergoing energy transfer to rhodamine
3. Excitation light 495 nm, emission at 576 nm: yielded intensity of the FRET or averaged distance between the dye molecules (forgery-proof)

Each of the prepared capsule types yielded an specific ratio from signal 1: signal 2 : signal 3. In case of measuring small differences in the signal intensity, already these both dyes are enough in order to produce a huge amount of codes. However the number of dyes in the capsules can be up to 7.

Filling the capsules with reactive macromolecules:

There are three different ways for immobilizing macromolecules in the interior:

1. Ship in bottle synthesis of polymers inside the capsules (Fig. 6).[12]
2. Switching the permeability of special capsules for the corresponding macromolecules by means of salt or pH (Fig. 7)[11]
3. Precipitation of an instable complex between the macromolecule and an auxiliary compound onto the templating colloid. Then encapsulation of the material by the usual LbL method and dissolution of the core and the macromolecule complex.[8]

Color-coded nanoparticles

Summary

Monodisperse spherical colloids were coated by means of the layer by layer method alternately with polyanions and polycations. The polycation was covalently linked (labelled) with fluorescence dyes, which absorbs and emit at different wavelengths. Per layer a defined amount of labelled polymer was deposited. The dye amount can be varied by the label content of the polymer or by co-assembling of unlabelled polymer. Different combinations of dye labels were assembled on the particles, separated from each other by intermediate layers of the polyanion. Undesired interactions between the dyes such as self-quenching, Förster Resonance Energy Transfer FRET or formation of H- or J- aggregates could be suppressed by assembling enough intermediate layers between the dyes. In turns the setting of defined distances (0 – 6 nm) between suitable dye pairs induces an FRET signal which can be controlled by the number of intermediate layers independent of the dye concentration. The coding can be read out by the fluorescence signal of the capsules at different excitation as well as emission wavelength. That could be Confocal Laser Scanning Microscopy for singular capsules, static fluorescence spectroscopy for immobilized capsules or flow cytometry for capsules in solution. A fluorescent activated cell sorter (FACS) allows the quantitative separation of the differently coded capsules. Furthermore the templating cores can be dissolved yielding hollow color-coded capsules. The interior of such capsules can be used for the immobilization of macromolecules (polyelectrolytes, proteins, enzymes) which can fish complementary compounds from the solution by physisorption, chemisorption or biological binding.

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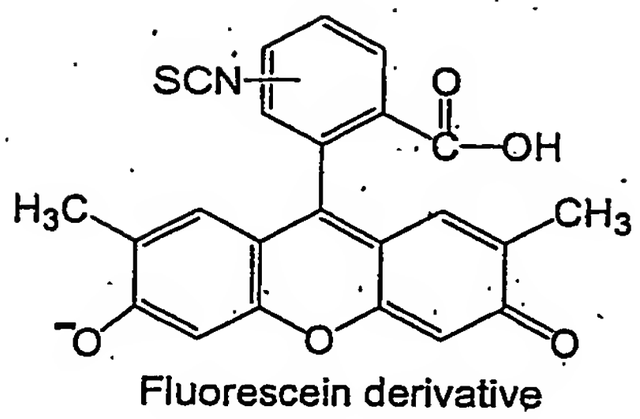
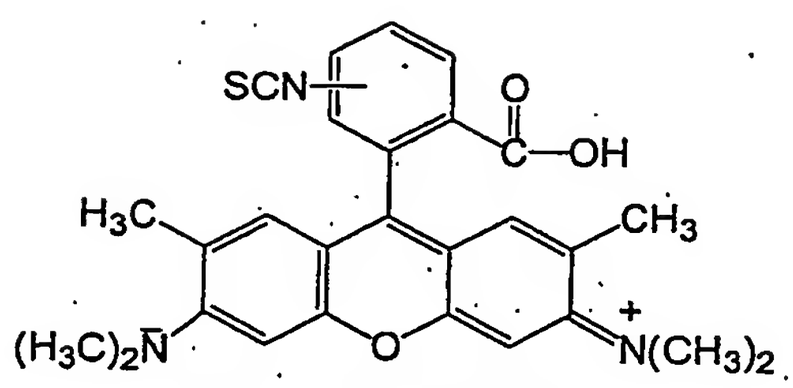
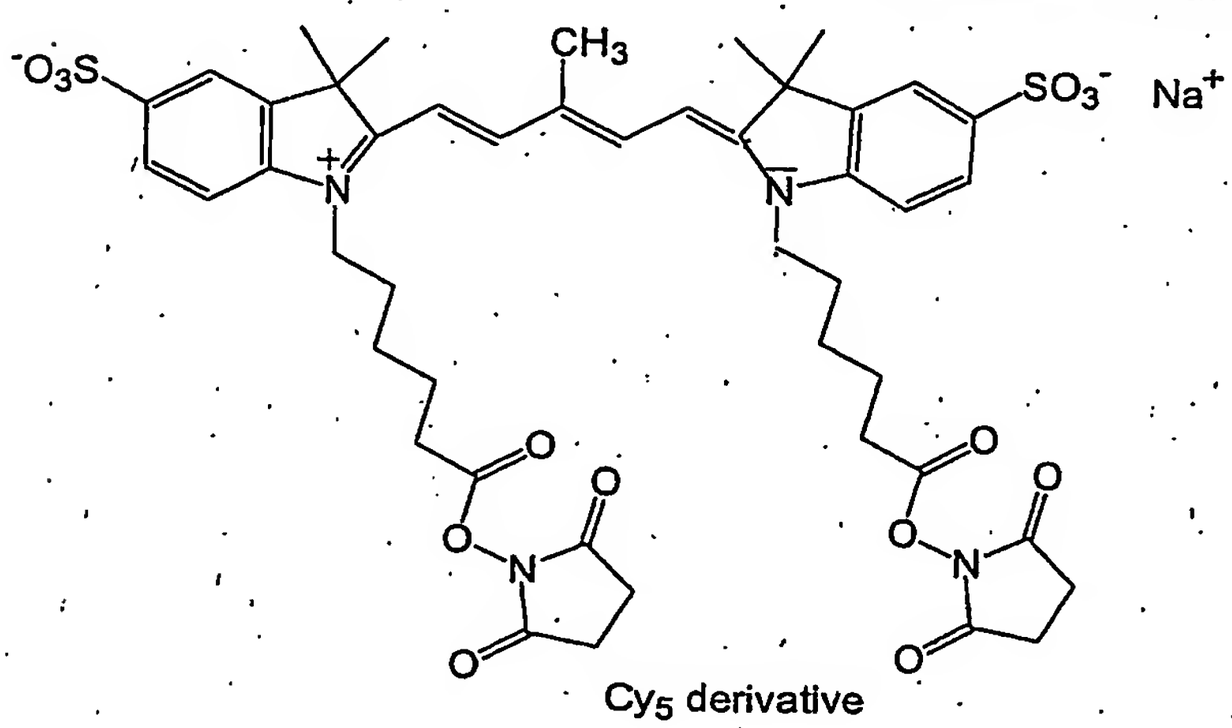


Fluorescein derivative

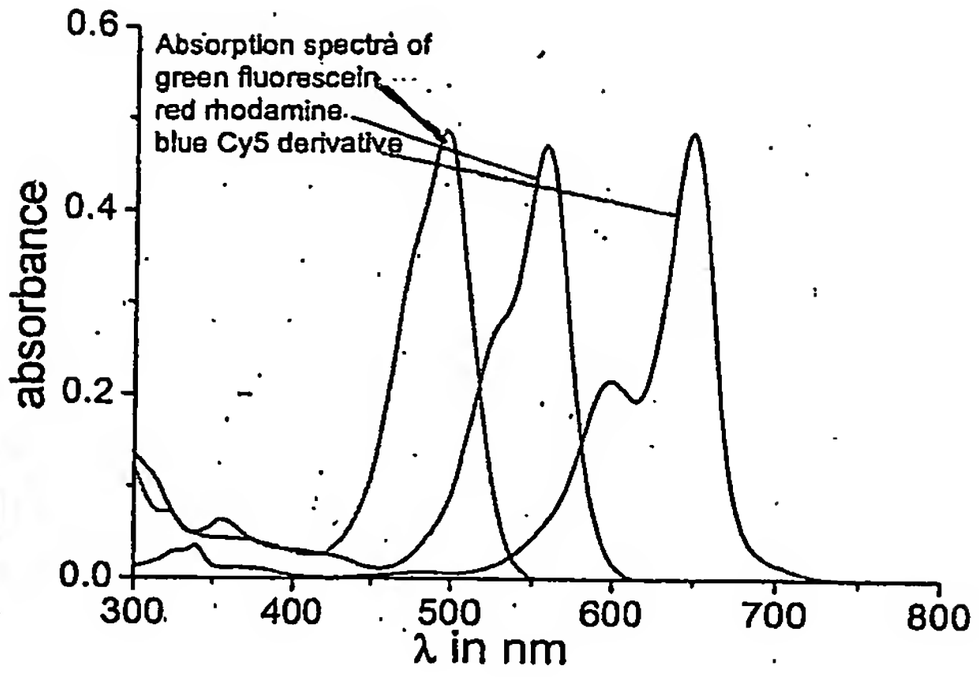


Rhodamine derivative

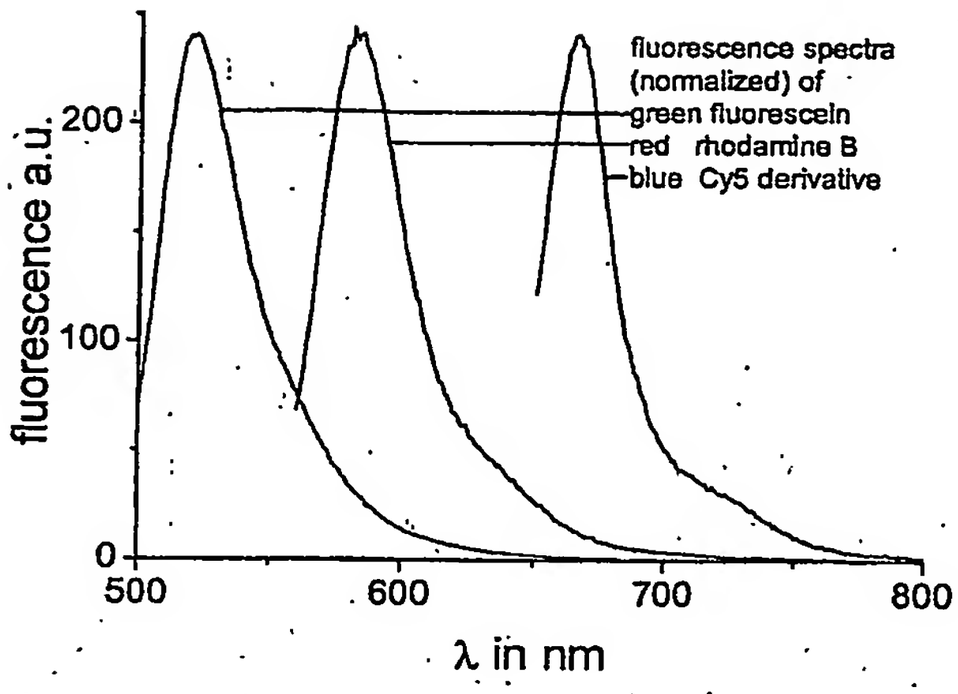


Cy_5 derivative

Fig. 1



a)

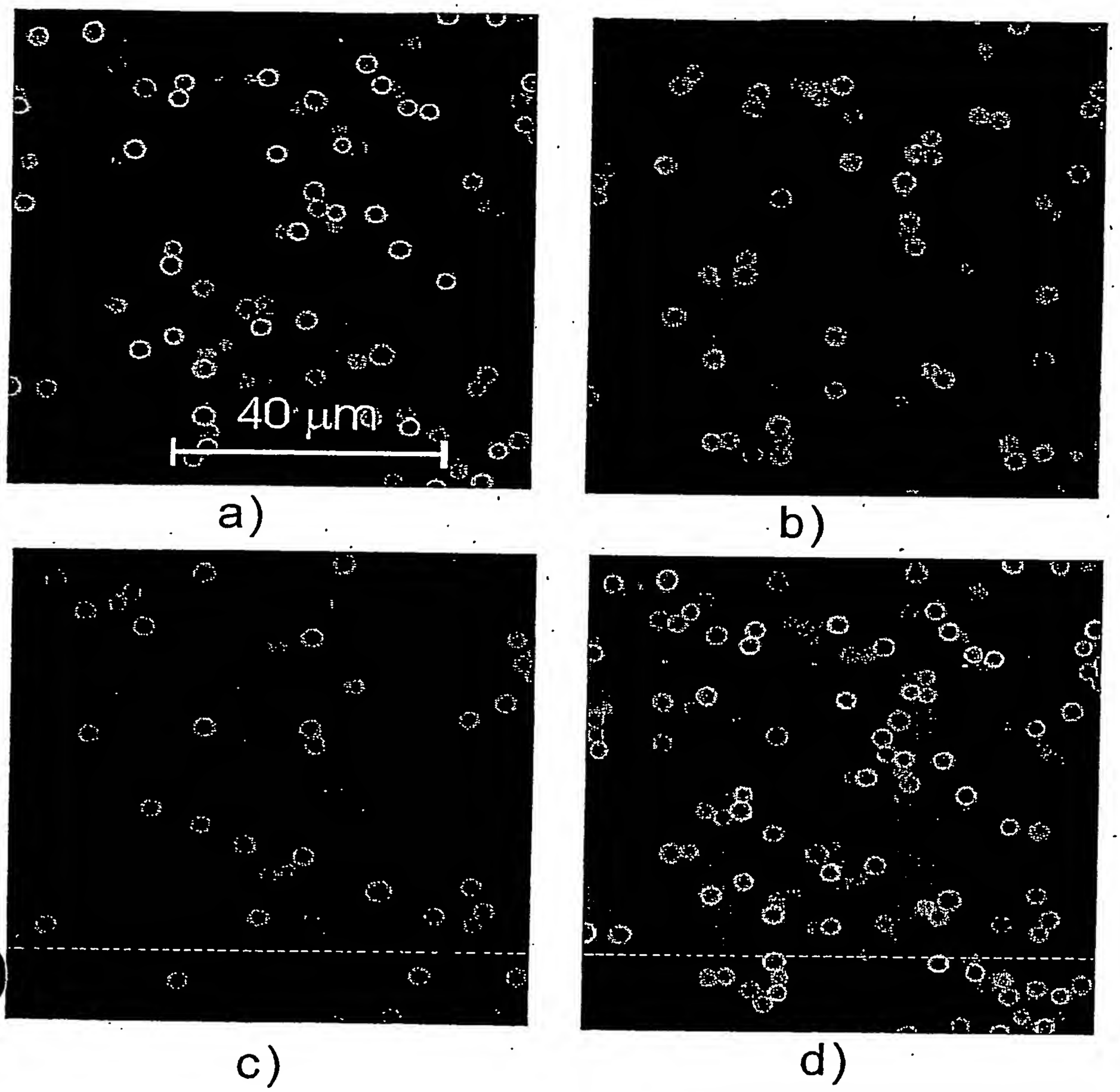


b)

a) absorption spectra of PAH-Fl, PAH-Rho, PAH-Cy5, intensities are normalized

b) fluorescence spectra of PAH-Fl, PAH-Rho, PAH-Cy5, intensities are normalized

Fig. 2



Confocal images of a mixed sample of the coloured capsules

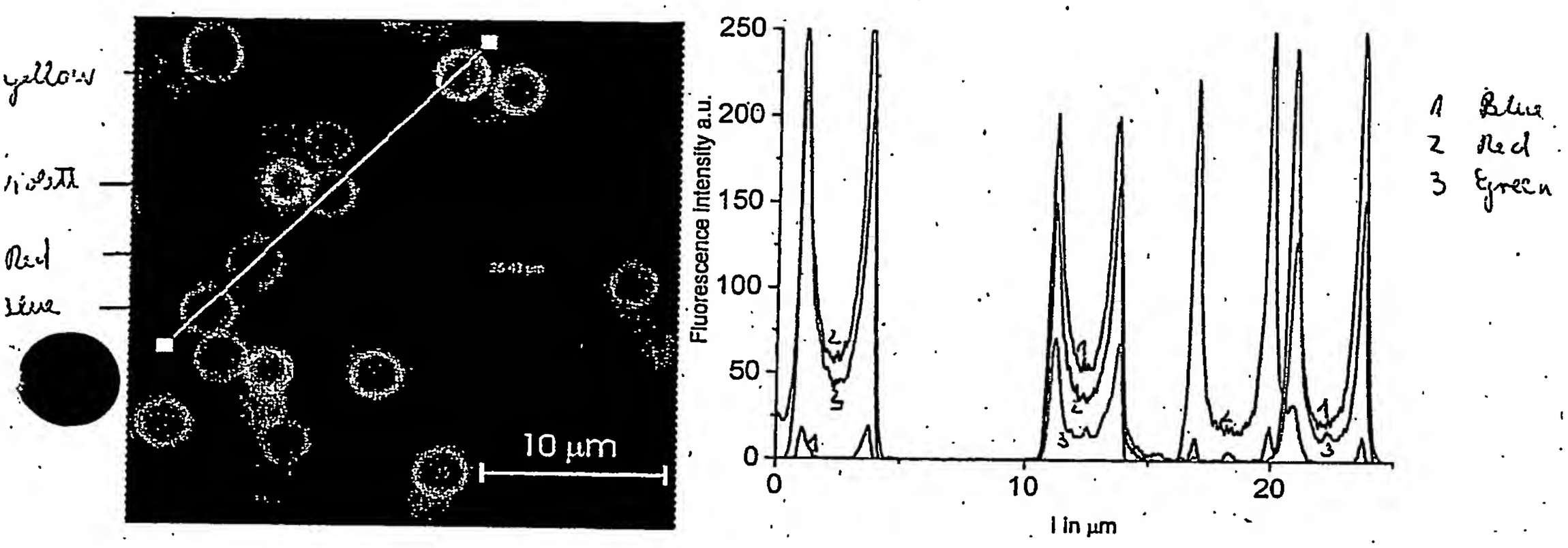
a) fluorescein channel

b) rhodamine channel

c) Cy5 channel

d) overlay of the three channels

Fig. 3

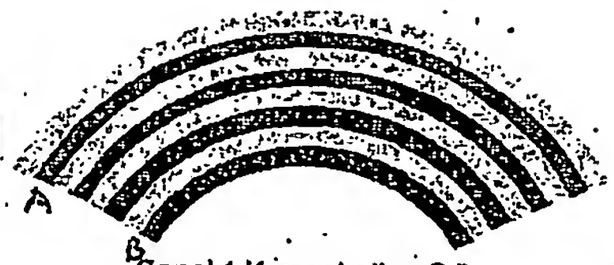


Confocal image of a mixed sample of coloured capsules 2, 7, 1, 5:

a) overlay image of the three colour channels

b) profile of fluorescence intensities along the white line in a)

Fig. 4



Signal 1 Konzentration Grün : 5
Signal 2 Konzentration Gelb : 5
Signal 3 FRET(Abstand Gelb/Grün) : 1

Signal 1 Konzentration Grün : 4
Signal 2 Konzentration Gelb : 5
Signal 3 FRET(Abstand Gelb/Grün) : 3

Signal 1 Konzentration Grün : 3
Signal 2 Konzentration Gelb : 5
Signal 3 FRET(Abstand Gelb/Grün) : 5

Signal 1 Konzentration Grün : 6
Signal 2 Konzentration Gelb : 5
Signal 3 FRET(Abstand Gelb/Grün) : 10

Signal 1 Konzentration Grün : 1
Signal 2 Konzentration Gelb : 5
Signal 3 FRET(Abstand Gelb/Grün) : 2,5

Signal 1 Konzentration Grün : 4
Signal 2 Konzentration Gelb : 2
Signal 3 FRET(Abstand Gelb/Grün) : 2,5

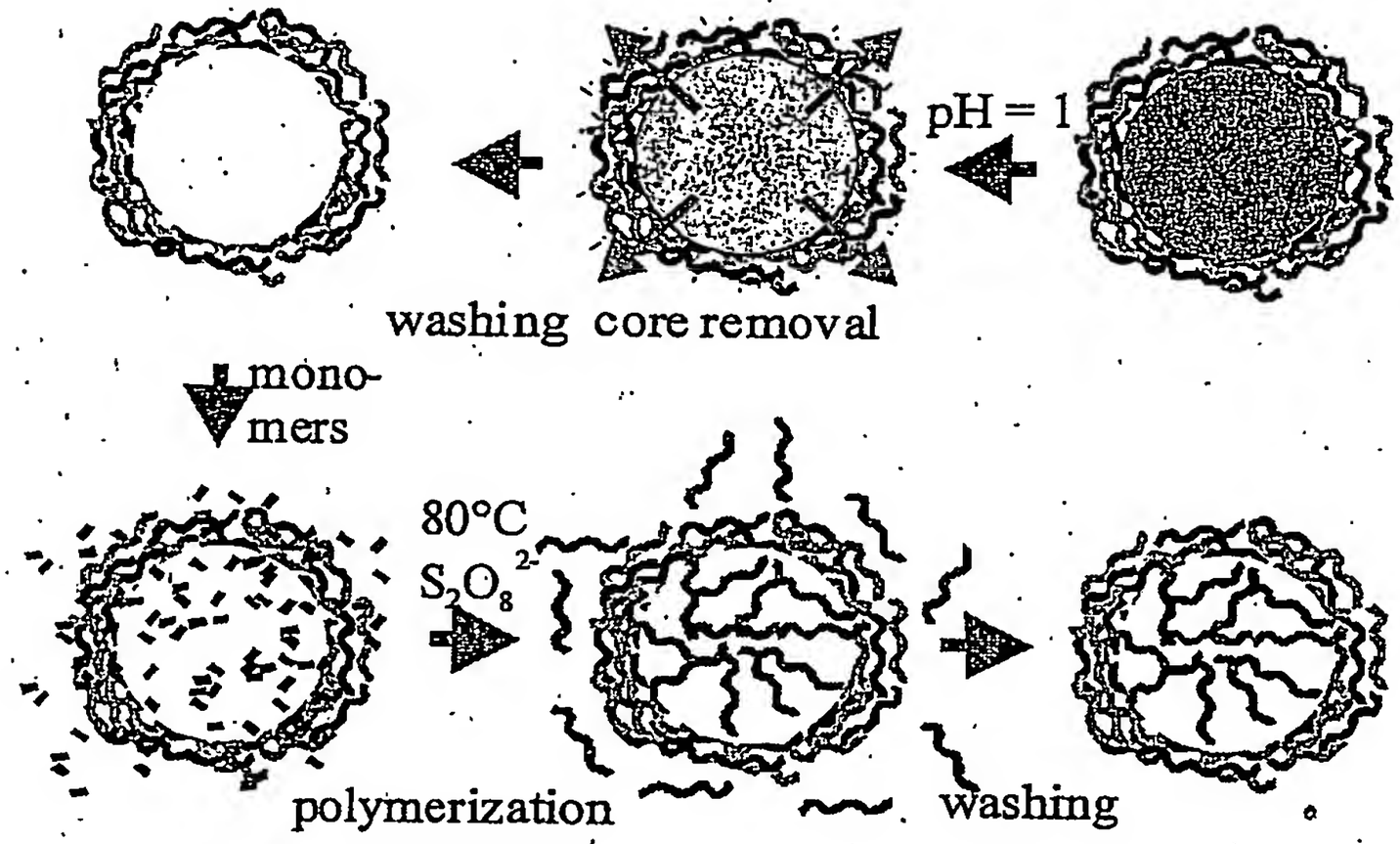
A Red
B Green

prepared layer combinations

a-c) different FRET signal intensities at same dye concentration

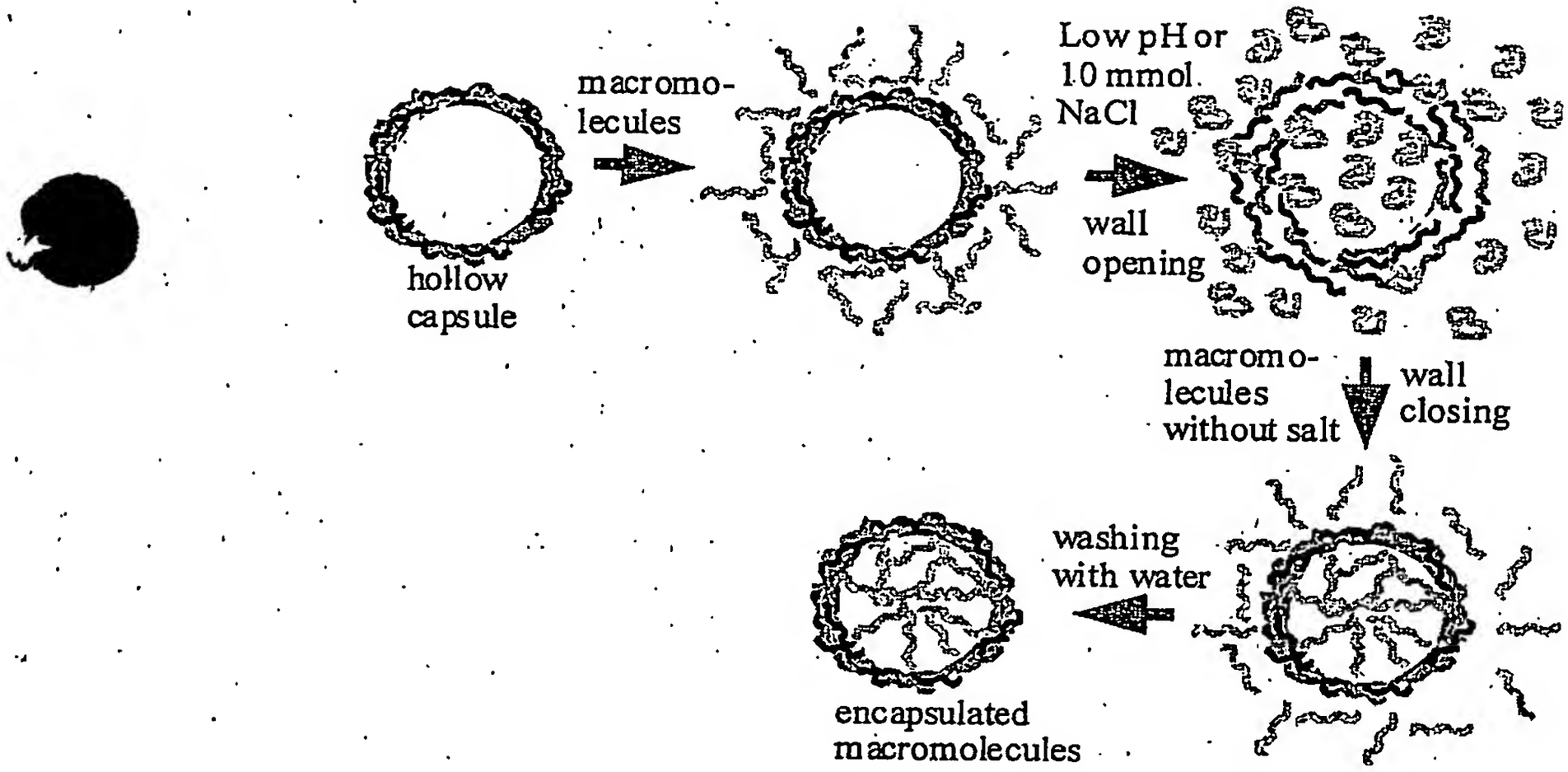
d-f) different FRET signal intensities at different dye concentration

Fig. 5



Ship in bottle synthesis of polymers inside the capsules

Fig. 6



Principle of loading MF capsules (8 layers) via switching the permeability of special capsules for corresponding macromolecules by means of salt or pH

Fig. 7